

AN INVESTIGATION OF THE VOLATILE ORGANIC
COMPOUNDS PRODUCED DURING FERMENTATION

by

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INTRODUCTION

Much has been said about the flavor of food products, but little concrete information is available concerning the actual constituents of flavor. The most obvious reason for this lack of knowledge is the complexity of the chemistry involved. The realization of the importance of flavor and the willingness to consider it from the standpoint of the chemical nature of the substances involved is a relatively new concept.

Early investigations with food dealt with various means of preservation and improving its appearance. Because of the need for food and the willingness to accept preserved foods, many foods were accepted which may not be entirely desirable today and may not be totally acceptable in the future. Bread is one of these foods. White bread has such little taste to the average person that flavor comparisons, even by a trained test panel, often are confused with other bread characteristics such as crumb texture or appearance. A variety of opinions exist concerning which flavor characteristics are most desirable in bread.

As with all processed foods, the flavor of bread is a function of both the ingredients and the processing. The complex of chemical and enzymatic reactions occurring during fermentation and during the oven baking process undoubtedly give rise to the formation of a number of volatile organic compounds which contribute to the flavor of the finished product.

This research is part of an extensive fundamental research program on bread flavor designed to find out what chemical compounds are present, in what quantities, and to what extent they are essential to bread flavor. Specifically, the objective of this work was to study the carbonyl components of bread flavor which are produced in a pre-ferment during fermentation.

REVIEW OF LITERATURE

Bread Flavor

Progress has been slow concerning knowledge of the constituents of a desirable bread flavor. Fisher and Halton, in 1938, (10) found that the "taste" of bread was improved as the pH of the dough was decreased. Acidic intermediates produced during fermentation could be detected by the odor of the dough which had undergone prolonged fermentation. This odor was attributed by various authors to acetic acid, butyric acid, propyl alcohol, butyl alcohol, amyl alcohol, and various esters, but no objective evidence concerning the presence of these compounds in bread had been recorded. Visser't Hooft and De Leeuw (46) concluded that bread flavor depended on the ingredients used as well as products formed during fermentation. They found that diacetyl was present in bread with a good flavor but absent in ill-flavored bread. Visser't Hooft and De Leeuw (46) concluded that acetylmethylcarbinol (acetoin) was a natural constituent of bread and that it was formed by the action of yeast on acetaldehyde during fermentation. Acetylmethylcarbinol was oxidized during fermentation to form diacetyl or dehydrogenated to form 2,3 butylene glycol. The rate of oxidation of acetoin was slow and the rate of evaporation of diacetyl was very fast, hence, no appreciable amounts of diacetyl accumulated in bread.

Maiden (30) found no improvement in the flavor of bread when acetylmethylcarbinol or diacetyl were added to a standard dough formula. When diacetyl was used at high rates, it produced an undesirable bread flavor.

Ingels, et al., (15) used a taste panel to determine the organoleptic characteristics of bread made by several formulas and representing several degrees of freshness. No conclusions were reached concerning the causes of

bread flavor, but it was accepted generally that freshness and flavor were positively correlated. They concluded that judging of odor and taste in bread was not a simple procedure since it required great care in preparation and control of the age of samples. Ingels, et al., (15) and Moir (33) believed that only people with a very keen sense of smell and taste should be eligible for a bread flavor panel.

In 1935, the United States Department of Agriculture appointed a committee (King, et al., 24) to study bread characteristics. This committee appointed a sub-committee whose objective was to investigate the factors which constitute a desirable bread flavor. The sub-committee carried out experiments involving various methods of mixing, formulas, flour grades, classes of wheat, bleaching effects, fermentation temperature, baking temperature and degrees of freshness. It was concluded by a taste panel that a rich formula as well as fresh bread contributed to good bread flavor but the method of this study was not sufficiently refined to detect small differences. Further observations revealed that large numbers of trained judges did not increase the validity of the experiments.

Cathcart (5) started experiments on bread flavor in 1935. His investigations were similar to those of King, et al., (24) and Ingels, et al., (15). Bread was prepared by varying one factor at a time and a panel of judges scored the bread according to odor and taste. Cathcart concluded that factors other than ingredients affected bread flavor. His conclusions were not specific because results varied with different sets of judges in different parts of the country.

Baker and Mize (1) baked crustless bread by making dough the resistance between two electrodes carrying alternating current. They found that the flavor of bread was due to the flavor of the ingredients plus products

developed by the yeast and products developed by heat reactions in the crust. The flavor products developed in the crust during baking reached the interior of the loaf and were believed to be involved in changes that occurred in the flavor of bread during aging and staling.

Luers (28) reported on the formation of flavor substances during fermentation of beverages. The aromatic substances produced differed depending upon the type of fermentation substrate, and were either transformed into other compounds or were volatilized during the later stages of fermentation or storage.

The characteristic aroma and flavor substances in beverages are not amenable to analysis mainly because they usually occur in minute concentrations which necessitate the use of large volumes for analysis. Luers partially solved this problem by means of extensive fractional distillation of large volumes of brew. Acetaldehyde and furfural were isolated. He postulated that the acetaldehyde was converted to acetoin via condensation. This theory was in agreement with that of Visser't Hooft and De Leeuw (46) and has been confirmed by the Embden-Meyerhof Scheme (Fruton and Simmonds, 11).

Baker, et al., (2) reported in 1953 that the fermentation of dough and formation of a brown crust were essential for satisfactory flavor. The analysis of an oven condensate obtained from bread baked after fermentation revealed the presence of ethanol, acetic acid, fusel oil, pyruvic aldehyde, diacetyl, furfural, and other iso-aldehydes, ketones and esters. Pence (35) also found alcohols, acids, esters and aldehydes and/or ketones to be present in baking oven vapors.

Wiseblatt (50) baked bread from a yeasted dough which was not given time to ferment but was leavened by chemical means. Such bread had acceptable grain and texture, but was deficient in taste and aroma. Thus, fermentation

was necessary to develop good bread flavor. Since some commercial processes for breadmaking carry out fermentation without flour, and continue to obtain desirable bread flavor, it is assumed that flour is of little importance for the development of flavor substances during fermentation.

It is generally considered that bread produced by the sponge dough method has better flavor than that produced by the straight dough method. Although there is some evidence that the physical treatment in mixing has something to do with flavor in bread, the differences in fermentation are probably chiefly responsible for the flavor differences between the two types of bread.

Wiseblatt (50) believed that many of the volatile flavorants formed during fermentation are probably too volatile to remain in bread after baking, except in trace amounts. Undoubtedly, some of the substances produced during fermentation undergo further reactions at oven temperatures. Thus, it becomes difficult to establish unequivocally the origin of any compound which is detected in bread or oven gases.

Wiseblatt (50) identified succinic, lactic, itaconic, hydrocinnamic and benzoic acids as well as their ethyl esters in the crumb of fresh bread and further investigations are underway. Johnson and Miller (21), using pre-ferments instead of bread crumb, found lactic, acetic, butyric and an unidentified acid.

Current studies at the American Institute of Baking are concerned with carbonyl compounds occurring in the crust of freshly-baked white bread. Steam-volatile carbonyl compounds have been converted into their 2,4-dinitrophenylhydrazones which are being resolved and identified by paper chromatography. Compounds already identified include furfural, diacetyl, pyruvic

acid, ethyl pyruvate, acetaldehyde, levulinic acid, and ethyl levulinate. All of these compounds, except levulinic acid, have pronounced odors, and, undoubtedly, are important contributors to the aroma of bread.

Column Chromatography of Carbonyl Compounds

Column chromatography has been used widely to study the derivatives of carbonyl compounds (36, 39, 44). Strain (44), working with the 2,4-dinitrophenylhydrazones of aldehydes and ketones, obtained satisfactory separation of these compounds when they were adsorbed and developed in Tswett columns of fibrous alumina or talc. The separation of the compounds could be followed because the 2,4-dinitrophenylhydrazones were colored compounds. Using the Tswett column, Wahhab (47) showed that furfurals could be separated from food products. In the same year Stadtman (42) described a more satisfactory method for separating, by column techniques, and identifying the 2,4-dinitrophenylhydrazone derivatives of carbonyl compounds. The 2,4-dinitrophenylhydrazones were identified by their absorption spectra. More recently, Roberts and Green (39) found it possible to separate qualitatively mixtures of the 2,4-dinitrophenylhydrazones of acetaldehyde, propionaldehyde, acetone, and methyl ethyl ketone when acetone and propionaldehyde were not present in the same mixture. They used silicic acid and supercel in two to one ratio.

Gordon, et al., (12) investigated a number of adsorbents, including silicic acid, supercel, silica gel, activated alumina, calcium carbonate, and a mixture of two parts of silicic acid (Mercks' precipitated silicic acid, through 100 mesh) and one part of Cellite analytical filter aid (Johns-Manville Co.), for efficiency in the separation of 2,4-dinitrophenylhydrazones of acetaldehyde and n-butyraldehyde. The last combination of adsorbents was most effective.

Paper Chromatography of Carbonyl Compounds

A number of investigators (3,4,6,9,12,13,16,25,29,32) have separated the 2,4-dinitrophenylhydrazones of aldehydes and ketones by paper chromatography. If the separation is quantitative, it is a simple matter to determine the separated hydrazones spectrophotometrically by virtue of their strong absorption in the visible or ultraviolet range. It is possible to identify the isolated derivatives by their characteristic R_f values, spectra, melting points, mixed melting points, and x-ray diffraction patterns.

Rice, et al., (38) were able to separate a number of aldehydes and ketones having carboxyl groups by using single phase solvents such as diethyl ether/petroleum ether. They also found that paper impregnated with silicic acid gave more positive separation than untreated paper.

Cavallini, et al., (6) investigated solvent systems for separation of keto acids. They found butanol to be more suitable than phenol, benzyl alcohol or ethyl acetate. Meigh (32) used the two phase solvent, methanol/heptane, to study the derivatives of aldehydes and ketones. The heptane was the mobile phase and the methanol phase was used to saturate the atmosphere. The resolved yellow spots of 2,4-dinitrophenylhydrazones were somewhat elongated and could be viewed more clearly by spraying the paper with aqueous 10 percent sodium hydroxide. The red-brown color obtained after spraying varied slightly with the compound and was sometimes an aid to identification. Keto acids and dicarbonyl compounds did not move with this system.

In 1952, using paper chromatography, Huelin (13) studied the volatile aldehydes and ketones of whole apples. The water/butanol system recommended by Cavallini, et al., (6) for separating the derivatives of keto acids was unsuitable for simple aldehydes and ketones whose derivatives traveled with the solvent front. The methanol/heptane solvent system of Meigh (32), however,

was suitable for separating aldehydes and ketones containing up to and including four carbon atoms. The usual R_f values for 2,4-dinitrophenylhydrazones of simple aldehydes and ketones were as follows: formaldehyde 0.35-0.40, acetaldehyde 0.50-0.55, propionaldehyde and acetone 0.70-0.75, butyraldehyde and methyl ethyl ketone 0.85-0.90. The R_f values of the 2,4-dinitrophenylhydrazones of aldehydes and ketones with the same number of carbon atoms did not differ significantly. The derivative of diacetyl traveled at the same rate as formaldehyde and slower than furfural, while glyoxal remained at the origin.

Newcomb and Reid (34) modified the separation of the 2,4-dinitrophenylhydrazones of carbonyl compounds by first treating the paper with dilute sodium disulfite solution and drying. Since aldehydes are more reactive with disulfite than ketones, the distribution of aldehyde derivatives was retarded. These workers used a heptane/chloroform/water (9:1:10, v/v) solvent system to obtain relatively good separation.

Lynn, et al., (29) found that previous methods (12,13,25) possessed serious limitations in their ability to separate homologs. Phenoxyethanol impregnated paper was employed as the stationary phase and heptane as the mobile phase. They obtained good separation using as much as 250 μ l. of a 2,4-dinitrophenylhydrazone in a single spot. It was necessary to use standard derivatives for comparison purposes because the solvent traveled off the end of the paper in the time required for most separations. Thus, R_f values could not be obtained with this solvent.

While investigating the chemical composition of tobacco smoke, Buyske, et al., (4) found that paper chromatograms treated with N,N-dimethylformamide and developed with n-hexane effected separation of the 2,4-dinitrophenylhydrazones of furfural, formaldehyde, acetaldehyde, propionaldehyde, acetone,

methyl ethyl ketone, diethyl ketone and butyraldehyde. These compounds were identified by comparative paper chromatography and by their ultraviolet absorption spectra. The solvent systems for the chromatographic separations of these 2,4-dinitrophenylhydrazones reported in the literature (29,32) were found to be unsatisfactory because of poor resolution of mixtures or excessive tailing or both. This method had the distinct advantage of possessing a high capacity for total solids without tailing.

Several solvents have been investigated recently by workers interested in flavor research. Klein and De Jong (26) obtained separation of the carbonyl derivatives having seven carbon atoms or more by employing paraffin oil as the stationary phase and dioxan/water (4:1,v/v) as the mobile phase. Wiseblatt (48) used the heptane/methanol solvent system to investigate the carbonyl compounds recoverable from the crust of fresh, white bread. The compounds studied were those extractable from crust with 50 percent aqueous ethanol and volatile with steam, and which reacted with 2,4-dinitrophenylhydrazine to yield water insoluble derivatives. Using the corresponding derivatives of known carbonyl compounds as reference standards, Wiseblatt (48) identified the following compounds: acetaldehyde, diacetyl, furfural, ethyl pyruvate, ethyl levulinate, pyruvic acid, levulinic acid. All of these compounds were considered to be implicated to some degree in the flavor of fresh bread crust. In a later report, Wiseblatt (49) stated that repetition of paper chromatography using the solvent system n-heptane/nitrobenzene/methanol (5:1:3,v/v) did not reveal additional compounds.

Working with roasted coffee, Clements and Deatherage (7) found that solvents containing dimethylformamide provided good separation of neutral carbonyl derivatives and tolerated relatively heavy applications of the sample. They also noted that addition of pyridine to the cyclohexane/

dimethylformamide system gave better separation of the slower moving derivatives.

A rapid paper chromatographic procedure for separating saturated aliphatic aldehyde 2,4-dinitrophenylhydrazones has been described by Ellis, et al., (9). C_1 and C_6 derivatives were separated by ascending development on propylene glycol-impregnated paper with skellysolve C fraction/methanol. Ascending development on vaseline-impregnated paper with aqueous methanol separated C_7 to C_{14} derivatives.

Gas Chromatography of Carbonyl Compounds

The principle of gas chromatography was suggested in 1941 by Martin and Synge (31). They reported that "the mobile phase need not be a liquid but refined separations of volatile substances should be possible in a column in which permanent gas is made to flow over a gel impregnated with non-volatile solvent in which the substances to be separated approximately obey Raoult's law." This suggestion was neglected until 1951 when James and Martin (18) presented convincing evidence that their previous suggestion was of a sound basis. Application of gas chromatography to the separation of volatile fatty acids was described. The separations obtainable by this method were essentially parallel to those obtained by distillation, but good results were achieved much more easily and it was possible to work with very small quantities. Less trouble from azeotrope formation was another advantage of this new method of separation because the concentration of the substances to be separated in the liquid phase was always low and it was possible to choose a liquid phase which associated with only one component of the azeotropes. Since that time, progress in the field of gas chromatography has been extremely rapid. Numerous papers have been published, symposiums have been held, and

several manufacturers have instruments in production.

James, et al., (19) demonstrated that ammonia and amines could be separated on celite columns containing a mixture of hendecanol and liquid paraffin as the liquid phase at a temperature of 78.6°C . with only slight overlap of the zones of the three methylamines. The amines emerged from the column in order of their boiling points.

James and Martin (18) described the technique for the analysis of other volatile materials in 1952. They used columns ranging in length from 4 to 11 feet with a diameter of 4mm. packed with celite and an appropriate liquid phase. When the liquid phase was DC 550 Silicone containing 10 percent w/v stearic acid, all normal and iso-acids from formic to hendecanoic could be separated. Other results included the use of silicone and liquid paraffin as the stationary phase to separate many primary, secondary, and tertiary aliphatic amines.

James and Martin (18) used this method to separate aliphatic amines with liquid paraffin as the stationary phase. Good separation of primary and tertiary or secondary and tertiary amines was possible because of the absence of hydrogen bonding in tertiary amines.

These workers recognized that a more general method of detection was necessary in order to identify all materials capable of being distilled. Two methods were suggested. The first method utilized the fact that the thermal conductivity of a gas was changed by the presence of small amounts of foreign substances. The instrument, a carthoerometer, usually consists of four channels containing fine platinum wires heated electrically and arranged to lie along the axis of the channels. When a gas passes down a channel, the wire is cooled to an extent dependent on the thermal conductivity of the gas and the resistance of the wire changes. By using two comparator channels along

which only the pure gas passes and by connecting the two running in a bridge circuit, it was possible to detect very low concentrations of material in a gas stream. Commercial instruments today employ hybrids of this type of detector. The second method takes advantage of the change in density of a gas stream caused by the presence of small amounts of volatile material. This detector was termed a gas density balance.

Employing the gas density balance as a detector, James and Martin (17) found that by using a long enough column almost any degree of separation was obtainable. It was concluded that gas chromatography should be capable of extension to all of those substances capable of being distilled even at a few millimeters of mercury pressure.

Ray (37) extended the work of James and Martin (17) on the separation of volatile fatty acids and bases. Application of this technique to analysis of mixtures of hydrocarbons, alcohols, ethers, esters, aldehydes and ketones on a column of celite impregnated with dinonylphthalate also proved successful. Several possible methods of detection were investigated. Of these, the most convenient, if not the most sensitive, was the measurement of thermal conductivity changes. Nitrogen carrier gas was used at a flow rate of 20 cc/min.

One disadvantage to gas chromatography today is the interference of water with a quantitative determination. In order to prevent interference of water in the quantitative separation of ether and alcohol, Ray (37) used the following procedure: A quantity (10 ml.) of the solution was measured into a small distilling flask equipped with a short fractionating column. The solution was distilled into an ice-cooled receiver until it was evident that only water was being distilled. Five ml. of carbon tetrachloride was added to the distillate followed by sufficient anhydrous potassium carbonate to absorb the water present. The upper carbontetrachloride layer was separated and a 50 ml. portion was injected into the chromatography column at 80°C. The flow rate

of the nitrogen carrier gas was 20 cc/min. Calibrations were then carried out by injecting measured amounts of the individual compounds as solutions of known concentration in carbontetrachloride.

The type of carrier gas used is also an important factor. Wiseman (51) studied separations of alcohols and esters using nitrogen and helium as the carrier gases. He found helium to give a greater sensitivity, particularly at higher temperatures, but with broader peaks and less resolving power than nitrogen. The choice of the mobile phase will depend on which is more important.

Hunter, et al., (14) analyzed aldehydes formed in the reaction between amino acids and ninhydrin by injecting a sample of the mixture into a 10 foot column filled with silicone and celite. Helium was used as the carrier gas at a flow rate of 23 ml./min.

Dimick and Corse (8) were among the first workers to use gas chromatography as a method to identify volatile flavor compounds in food and food products. They recognized that the column packing was of utmost importance. Their substrate had a low vapor pressure at elevated temperatures and was thermo-stable. Silicone oil and carbowax were found to be the most practical. The temperature of the column was 150°C. and the helium flow rate was 62 ml./min. The following table indicates that different classes of compounds could be separated effectively:

Table 1. Residence time of volatile compounds (8).

Compound	Boiling Point °C.	Residence Time			
		Silicone		Carbowax	
		Min.	Sec.	Min.	Sec.
Ethyl Acetate	77.1	4	20	5	20
Ethyl Alcohol	78.3	1	30	8	0
Methyl Ethyl Ketone	80.0	4	40	6	0
Benzene	80.0	7	10	7	0
n-Heptane	98.0	10	20	1	30
Water	100.0	1	40	17	0

Other workers (20,43) have studied volatile flavorant material using gas chromatography to separate the components. General results follow closely those of earlier workers and a usual conclusion is that gas chromatography is basically the best technique available for such studies.

MATERIALS AND METHODS

Since study involving the chemistry of flavor is such a new concept, it was logical to assume that a variety of techniques existed for the solution of such a problem. A thorough literature review confirmed this assumption. The techniques used in this research were similar to those found in the literature (13,23,43,50).

Two categories of compounds were studied. These were (a) the volatile organic compounds released during fermentation, and (b) the volatile organic compounds remaining in the pre-ferment after fermentation.

Preparation of Carbonyl Derivatives

Preliminary investigations were designed to reveal the specific carbonyl compounds which were released during a six hour fermentation period of a pre-ferment. All fermentations were carried out at 30°C. This study required the preparation of 2,4-dinitrophenylhydrazones of the pre-ferment exhaust gases and of several standard carbonyl compounds.

A revision of the method employed by Phippen, et al., (36) was used to prepare the 2,4-dinitrophenylhydrazones of acetaldehyde, propionaldehyde, n-valeraldehyde, acetone, acetoin, diacetyl and furfural.

Nitrogen was passed, for one hour, through a solution containing the following ingredients: water, 290 ml.; sodium chloride, 4 g.; ethanol, 8 ml.; lactic acid, 20 ul.; carbonyl compound, 10 millimoles. This formula was

derived by considering the expected components of a pre-ferment during fermentation. The exit gases were passed through 460 ml. of 2,4-dinitrophenylhydrazine reagent. The orange precipitate formed in the reagent was then filtered, washed with distilled water and recrystallized from hot ethanol until melting points agreed with those found in the literature (41).

Derivatives of the carbonyl compounds which were released from the pre-ferment during fermentation were prepared by allowing the carbon dioxide, which was produced by action of yeast on sugar, to pass into 460 ml. of 2,4-dinitrophenylhydrazine reagent. The reagent was maintained at 0°C. in order to preserve volatile components. The 2,4-dinitrophenylhydrazones were then filtered, washed with water and recrystallized from hot ethanol three times. The standard pre-ferment formula was increased by a factor of 30 so that sufficient quantities of the derivatives were accumulated in the reagent.

The 2,4-dinitrophenylhydrazine reagent was prepared according to the procedure of Shriner and Fuson (41). Two grams of 2,4-dinitrophenylhydrazine were dissolved in 15 ml. of concentrated sulfuric acid. The solution was then added, with stirring, to 150 ml. of 95 percent ethyl alcohol, and the resulting mixture was diluted to 500 ml. with distilled water. It was then mixed thoroughly, filtered and stored in the refrigerator.

The 2,4-dinitrophenylhydrazones used in this research were all solids. In order to allow transfer of exact quantities, solutions were prepared by dissolving one ug. of 2,4-dinitrophenylhydrazone in one ul. of hot ethanol. Solutions were stored in small sample vials until needed for paper electrophoresis or paper chromatography. The transfer was made by means of a micro-pipette.

Preparation of Pre-Ferments

The formula of the standard pre-ferment used in this research is recorded in Table 2. The ingredients were mixed in a Waring Blendor for one minute and placed in a five gallon jug. The mixture was allowed to ferment for six hours at 30°C.

Table 2. Pre-ferment formula (40).

Ingredients	Quantity of Ingredients	
	:	(grams)
Water	:	320.0
Glucose	:	21.0
Malted Wheat Flour	:	1.8
Sodium Chloride	:	7.0
Brew Buffer ¹	:	2.1
Compressed Yeast	:	14.0

¹Standard Brands Inc., New York 22, N.Y. Contains calcium carbonate, ammonium chloride, wheat flour, calcium sulfate and sodium chloride. The brew buffer furnishes nitrogen and mineral nutrition which the yeast requires during the brew fermentation. It also controls acidity and thus eliminates the use of milk in the brew. Omission of the milk from the brew also lessens the chance for adverse bacterial action.

Paper Electrophoresis of Carbonyl Derivatives

Apparent separations are possible on the paper electropherogram if two substances are moved by the electrostatic flow and retarded to a different degree by adsorption on the paper. Whenever this is the case, the degree of separation may vary considerably with the potential and technique used (3).

Paper electrophoresis was the first method of analysis used to study the carbonyl compounds released during fermentation. The procedure for paper electrophoresis was similar to the method described by Tauber (45). Eight determinations were made simultaneously using Whatman No. 1 paper strips especially fitted for the system. The Spinco Model R Paper Electrophoresis system was operated at 185 volts and 11 MA. Two liters of barbital buffer

solution were poured over the paper strips, making sure the paper strips were in contact with the paper wick. The system was allowed to stand for 15 minutes until the closed cell became saturated with vapor, and then equal amounts of the standard 2,4-dinitrophenylhydrazones were spotted on the paper strips with a micro-pipette. Quantities of the samples ranged from 20 μ l. to 100 μ l. After development periods ranging from 6 to 14 hours, the strips were dried in a pre-heated oven at 120-130°C., and then sprayed with a 10 percent solution of potassium hydroxide.

Although several revisions were made in technique and method, the results obtained with paper electrophoresis were consistently negative. The carbonyl derivatives which had been prepared for paper electrophoresis were then subjected to paper chromatography.

Paper Chromatography of Carbonyl Derivatives

Both ascending and descending uni-dimensional chromatograms were made and various types of filter paper, sample sizes and development periods were explored. The following solvent systems were investigated:

1. Propanol/petroleum ether (65:35, v/v) (3)
2. Ethanol/petroleum ether (4:1, v/v) (3)
3. Anhydrous methanol/heptane (1:1, v/v) (3)
4. Butanol/ethanol/water (4:1:5, v/v) (3)
5. Fifteen percent ethyl acetate in ethyl ether (3)
6. Methanol/heptane (3:1, v/v) (32)
7. Heptane/methanol (2:1, v/v) (13)
8. Heptane/nitrobenzene/methanol (5:1:3, v/v) (48)
9. Cyclohexane/dimethylformamide (7)

Heptane/nitrobenzene/methanol (5:1:3, v/v) gave the best separation of the

carbonyl derivatives and was used extensively to study the compounds being released during fermentation.

Gas Chromatography

With the acquisition of a Perkin-Elmer Model 154-C Vapor Fractometer, attention was turned to the study of volatile organic fractions remaining in the pre-ferment after fermentation. This investigation was designed to separate and identify each volatile component of the pre-ferment. Gas chromatography was selected in preference to paper chromatography for this analysis because it was reportedly more sensitive, and trace amounts could be detected.

Collection of Distillate for Gas Chromatography. Two methods of collecting samples of the volatile fractions were investigated. First, a vacuum distillation of the pre-ferment was accomplished with an apparatus similar to the one described by Stahl, et al., (43). The objective of vacuum distillation of the pre-ferment was to allow a crude separation of widely boiling components so that, when injected into the chromatographic column, finer separations could be achieved. Two liters of the pre-ferment were vacuum distilled under a pressure of approximately 10 mm. The temperature was held constant at 40°C. by a vari-vac. The apparatus included a three liter flask, a ten inch fractionating column, a condensor and two condensate traps. All fittings were of the glass sintered type to avoid accumulation of foreign materials from rubber or cork. One of the condensate traps was designed to collect the majority of the water. It was water cooled. The other trap, maintained at 0°C. by an acetone-Dry Ice bath, was designed to collect the volatile organic fraction.

After several distillation trials, this method was discontinued because excessive amounts of water in the distillate interfered with proper separation

in the fractometer. The second method of collection involved selective solvent extractions.

Preparation of Extracts for Gas Chromatography. The pre-ferment was passed through a Sharples centrifuge at 18,000 r.p.m. to eliminate the yeast cells. The resulting solution was divided into 2500 ml. portions and each portion was extracted three times with a water insoluble solvent. The solvents investigated were benzene, ethyl ether, carbontetrachloride, and n-hexyl alcohol. The benzene, carbontetrachloride and n-hexyl alcohol extractions were accomplished with 150 ml., 100 ml., and 50 ml. portions, respectively, on separate pre-ferments, while ether extractions were carried out with 250 ml, 200 ml., and 150 ml. portions of the solvent.

Identification of Volatile Fractions. After the components of the pre-ferment were separated, identification became the major problem. Peaks were first identified by the addition of supposed constituents as suggested by Keulemans (23). This method was used successfully to identify five components of the pre-ferment. If the sample was mixed with a pure compound that was suspected to correspond with a certain peak, and a new chromatogram was run, the height of the peak would be increased if the supposition was correct, while a new peak or a break in the old peak would result if the two compounds were not identical. Two stationary liquids of differing polarity were employed to preclude the probability that the substance added might have had the same retention time as another compound. The possibility of this happening was not likely, but it was existent.

Attempts to confirm qualitative identification of each individual fraction by means of a Perkin-Elmer Model 137 Infracord Spectrophotometer were not successful. Failure to accomplish further identifications by this method was attributed to the fact that all components were present in concentrations less than .1 percent.

Quantitative Analysis. The actual quantitative interpretation of the differential gas chromatograms was based on peak height and peak area. Peak areas were determined by multiplying the peak height by the width at half height (23). Calibration curves were made for each of the five components identified. The benzene extracts were used exclusively for quantitative analysis because they contain all the volatile components in question.

A Study of the Effect of Microorganisms on Flavor. Further work included a comparison of the volatile organic fractions present in the pre-ferment after fermentation when various microorganisms had been added to the pre-ferment. The microorganisms presented in Table 3 were studied. These microorganisms had previously been associated with flavor differences in bread by Robinson, et. al., (40).

Table 3. List of known organisms used as flavor producers.

Organisms	Source
<u>Aerobacter aerogenes</u> T.F.I.	Prof. V. C. Foltz, K.S.C.
<u>Bacillus pumilus</u>	Pre-ferment
<u>Lactobacillus plantarum</u>	Pre-ferment
<u>Pediococcus cerevisiae</u> 10791	American Type Culture Collection
<u>Propionibacterium</u>	Dept. of Bacteriology, K.S.C.

Each microorganism to be tested for flavor production was cultured in 10 ml. of sterile, enriched, nutrient broth. A second 10 ml. of sterile, enriched, nutrient broth was inoculated with these cultures and incubated for 24 hours at 30°C. Four 100 ml. portions of sterile, enriched, nutrient broth were inoculated with this second 10 ml. inoculum. After a 24 hour incubation period at 30°C. the volume of each 100 ml. inoculum was increased to 500 ml. with sterile, enriched, nutrient broth. After a 72 hour incubation period at 30°C. these 500 ml. portions of cultures were combined and used to replace two liters of water in a pre-ferment mixture for flavor analyses.

RESULTS AND DISCUSSION

A Study of the Volatile Organic Compounds Released
During Yeast Fermentation of a Pre-Ferment

Several attempts to produce positive evidence regarding the volatile organic compounds involved in fermentation of pre-ferments were made. The sequence of investigation was dependent upon availability of instruments and supplies. The first objective of this research was to investigate the 2,4-dinitrophenylhydrazones of the carbonyl compounds released during fermentation.

The standard derivatives of acetaldehyde, propionaldehyde, n-valeraldehyde, acetone, acetoin, and furfural were subjected to paper electrophoresis following the method of Tauber (45). The final results after drying and spraying the strips with a 10 percent solution of potassium hydroxide indicated virtually no movement of the 2,4-dinitrophenylhydrazones. The experiment was repeated several times using various concentrations of the derivatives and various instrument conditions. At the end of a 14 hour development period the paper strips were treated as before with no positive results. Failure to obtain movement of the derivatives by paper electrophoresis, together with the fact that paper chromatography was a more widely recognized technique for the study of 2,4-dinitrophenylhydrazones, indicated that emphasis should be placed on paper chromatography. This type of analysis had been used successfully to study 2,4-dinitrophenylhydrazones by many workers (13, 29,32).

After preliminary investigation of solvent systems, it was found that the methanol/heptane (1:1, v/v) offered best separation of all 2,4-dinitrophenylhydrazones involved. Further work with this solvent system revealed that a heptane rich solvent heptane/methanol (2:1, v/v) was more suitable and

heptane/nitrobenzene/methanol (5:1:3, v/v) as suggested by Wiseblatt (48) was the most effective of any. Further study was confined to this solvent system.

Whatman No. 1, 2, 3 and 4 filter papers were used, but No. 1 and 3 papers offered best separation of the 2,4-dinitrophenylhydrazones. Experimentation with sample size indicated that 20 ul. of the 2,4-dinitrophenylhydrazone solution gave optimum separation.

Employing the heptane/nitrobenzene/methanol (5:1:3, v/v) solvent system, 20 ul. samples of the various 2,4-dinitrophenylhydrazones were spotted on 12 x 24 inch sheets of Whatman No. 1 and 3 filter papers. A 12 x 24 inch borosilicate glass jar served as a chromatography chamber and the 15 hour development period was carried out in a dark room to minimize temperature and light effects. Plate I, an example of the resulting chromatograms, shows that the pre-ferment derivative had an R_f value equal to that of acetaldehyde. This observation indicated that acetaldehyde was the only carbonyl compound released in sufficient quantities to be detected by this method of analysis. Acetaldehyde was expected to be released in larger quantities than other carbonyl compounds because it was the only one examined which had a boiling point below 30°C. It is possible that other carbonyl compounds which exert a positive vapor pressure at 30°C. could have been released in quantities too small for detection by this method. It is also feasible that carbon dioxide could remove carbonyl compounds from the pre-ferment. In an effort to detect the release of other carbonyl compounds, the gases from 27 liters of pre-ferment were bubbled through 460 ml. of 2,4-dinitrophenylhydrazine reagent. Paper chromatography revealed that the pre-ferment derivative again did not separate and that it had an R_f value identical to that of acetaldehyde.

Evidence presented in the literature regarding other derivatives of carbonyl compounds such as semicarbozones indicated that the physical properties of such derivatives would preclude their use in paper chromatography.

PLATE I

Paper chromatogram of 2,4-dinitrophenylhydrazones of the following carbonyl compounds: A. Acetaldehyde; B. Propionaldehyde; C. n-valeraldehyde; D. Acetone; E. Acetoin; F. Furfural; G. Pre-ferment.

Solvent system: Heptane/nitrobenzene/methanol
(5:1:3, \sqrt{v})

Filter Paper: No. 3 Whatman

Development Time: Fifteen hours

PLATE I



A

B

C

D

E

F

G

Further work employing a Perkin-Elmer Model 154-C Vapor Fractometer confirmed the release of acetaldehyde during fermentation, and indicated that ethyl alcohol and ethyl acetate were released in trace amounts. The exhaust gases from a pre-ferment fermentation carried out at 30°C. were passed into carbontetrachloride which was maintained at 0°C. by means of an acetone-Dry Ice bath. Twenty ul. of the carbontetrachloride were then injected into the fractometer which was equipped with Column "F". The separation was carried out at 96°C. with a helium flow rate of 40 ml/min. The resulting chromatogram contained peaks at 2.4 minutes, 3.2 minutes and 5.1 minutes, which were more intense when acetaldehyde, ethyl alcohol and ethyl acetate, respectively, were added to the extract. The production of these compounds during fermentation was due to the action of yeast on dextrose.

A Study of the Volatile Organic Compounds Remaining in the Pre-Ferment after Yeast Fermentation

The volatile organic compounds remaining in the pre-ferment after a six hour period of fermentation were studied by means of gas chromatography. Two methods of sample collection were employed to obtain volatile organic fractions for this study.

Vacuum Distillation. The sample accumulated in an acetone-Dry Ice trap during vacuum distillation of a pre-ferment was dried according to the procedure of Ray (37). Several peaks were formed when a 20 ul. portion of this material was injected into the fractometer. Although the number of peaks present were inconsistent, qualitative identification of acetaldehyde and ethyl alcohol was possible by adding small amounts of the known component and then observing an increase in peak intensity. These results were consistent when two ul., 20 ul., and two ml. samples were injected. The intensity of each peak increased with sample size.

Although these observations were significant qualitatively, the temperature (40°C.) and pressure (10 mm.) of the distillation were believed to have a deteriorating effect on the volatile components. Consequently, no quantitative analysis was attempted. Similar methods have been used to study flavorant compounds present in strawberries (36), but a much more elaborate apparatus was used.

Selective Solvent Extraction of the Pre-Ferment. Extractions were carried out with benzene, carbontetrachloride, ether and n-hexyl alcohol, maintaining a temperature below 30°C. in order to preserve the volatile components of the pre-ferment. Organic fractions of the benzene extracts were concentrated by freezing out the benzene (5.6°C.); however, further concentration of the extracts obtained with ether, carbontetrachloride, and n-hexyl alcohol was hindered by the apparent low freezing points of these solvents (-128°C., -23°C., and -51.6°C., respectively). Analysis by gas chromatography showed the benzene extracts to contain more organic fractions than extracts obtained by use of the other solvents.

Although benzene extracts gave the most desirable information, ether extracts revealed areas on the chromatogram which were covered up by the benzene peaks. When used in conjunction with one another, the benzene and ether extracts represented the best possible means of studying the volatile organic compounds present in the pre-ferment after fermentation.

Analysis of Extracts by Gas Chromatography - (Column "F"). Perkin-Elmer Column "F" was first used with a helium flow rate of 40 ml/min. and a temperature of 96°C. to separate the volatile organic fraction of the pre-ferment. Various size samples were injected into the fractometer and the separation of the fractions studied. Plates II and III represent sample chromatograms resulting from benzene and ether extracts, respectively. Seven components

PLATE II

Gas chromatogram of pre-ferment benzene extract using Column "F". A. Air; B. Acetaldehyde; C. Unknown compound; D. Unknown compound; E. Ethyl alcohol; F. Benzene; G. Unknown compound (high boiling fraction).

PLATE II

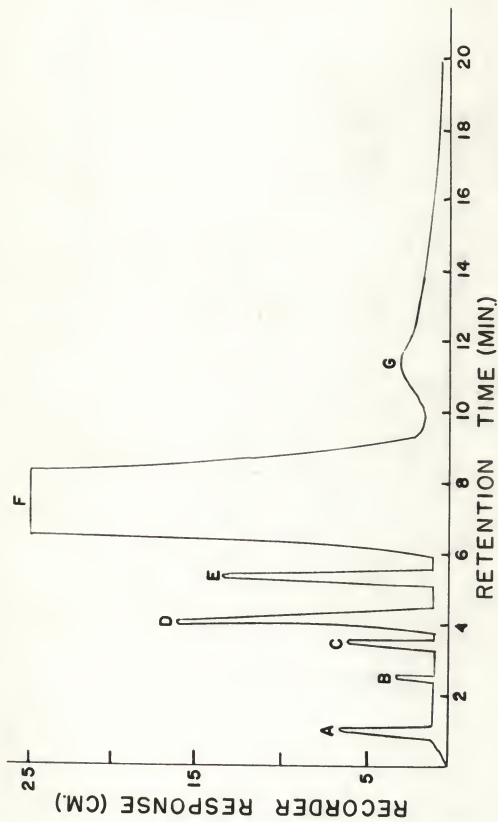
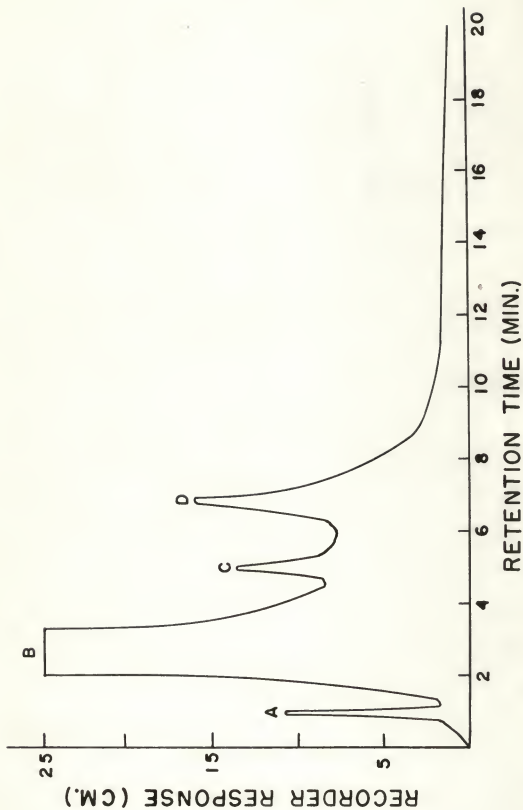


PLATE III

Gas chromatogram of pre-ferment ether extract using
Column "F". A. Air; B. Ether; C. Ethyl alcohol; D.
Unknown compound.

PLATE III



were observed in the benzene extract, and four were present in the ether extract. These results showed that the benzene and ether extracts were complementary and a complete picture of the fractionation was available only when both were used in conjunction with one another.

Identification of Peaks by Adding Supposed Constituents. Identification of the seven components present in the benzene extract was first attempted by adding micro amounts of a pure supposed constituent and observing the increase in peak intensity. The largest peak at 7.6 minutes was identified as benzene. The peak at 2.3 was increased in intensity when pure acetaldehyde was added to the extract, and the peak at 5.2 minutes was increased in intensity upon addition of a pure sample of absolute alcohol. Other compounds which might conceivably be present in the pre-ferment were investigated, but no further identification was possible.

When lower column temperatures were used (75°C.) the peak at 3.9 separated into two peaks, but no further identification was possible.

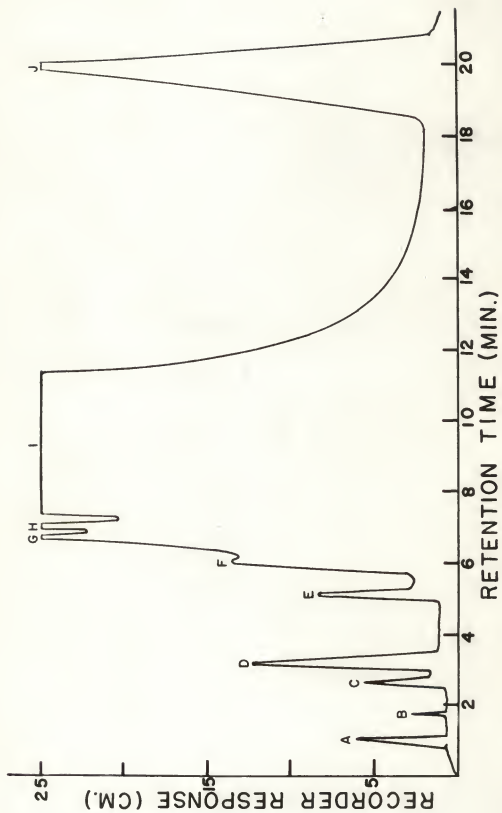
Analysis of Extracts by Gas Chromatography - (Column "A"). Analysis of the benzene extract with Column "A" allowed more extensive identification of the existing peaks. The separation was generally by boiling point and the peaks varied more in retention time than they did with Column "F".

For this analysis, 20 ul. of the benzene extract were injected into the column, employing a temperature of 95°C. and a flow rate of 40 ml/min. Plate IV shows that five volatile components of the pre-ferment extract were identified. They were acetaldehyde (.03 percent), ethyl alcohol (.1 percent), ethyl acetate (.06 percent), diacetyl (.06 percent) and lactic acid (.1 percent). The method of "addition of supposed constituents" was used to make these identifications. All of these compounds have previously been associated with alcoholic fermentation (22,28) and their presence in a pre-ferment can be explained by the Embden-Meyerhof scheme (11).

PLATE IV

Gas chromatogram of pre-ferment benzene extract using Column "A". A. Air; B. Acetaldehyde; C. Unknown compound; D. Ethyl alcohol; E. Ethyl acetate; F. Impurity in benzene; G. Diacetyl; H. Impurity in benzene; I. Benzene; and J. Lactic acid plus other high boiling compounds.

PLATE IV



Studies Involving the Effects of Microorganisms on Flavor Production.

Benzene and other extracts obtained from per-ferments inoculated with Pacillus pumilus, Lactobacillus plantarum, Aerobacter aerogenes T.F.I., Pediococcus cerevisiae 10791 and Propionibacterium, respectively, were analyzed with the vapor fractometer. Columns "A" and "F" were employed with a helium flow rate of 40 ml/min. and a column temperature of 95°C.

Results indicated that the presence of Aerobacter aerogenes or Pediococcus cerevisiae in the pre-ferment would increase the concentration of diacetyl in the extracts. The other microorganisms tested did not have a marked influence on the quality or quantity of components present in the pre-ferment extracts. Fruton and Simmonds (11) reported that pyruvic acid is transformed to acetoin during yeast fermentation by a variety of microorganisms. The increased amounts of diacetyl produced when certain microorganisms are added to the pre-ferment are probably a result of more pyruvic acid being transformed into acetoin and a subsequent conversion of the acetoin to diacetyl via oxidation (28).

Infra-red, as an Aid in Identification. Further identification work was based on the possibility of gaining knowledge about the specific groups present in each fraction of the extract by means of a Perkin-Elmer Model 137 Infracord Spectrophotometer. The Perkin-Elmer Vapor Fractometer was equipped with a gas sampling device whereby each fraction could be collected as the representative peak was formed by the recorder. The compound which yielded the peak with the greatest area was explored first.

Three collection techniques were investigated. First, a sample of the gas responsible for the largest peak was forced to displace a volume of air over mercury. This gas sample gave no response when analyzed by the spectrophotometer. The same results were evident when the gas sample was condensed

in an acetone-Dry Ice trap. A third technique also failed to give response when the gaseous fraction was allowed to pass into a small volume of carbon-tetrachloride which was maintained at 0°C. These techniques were believed to be unsatisfactory because the quantity of sample available was insufficient to give a significant response in the spectrophotometer.

Identification with a Large Diameter Column. In an attempt to allow greater volumes of each sample to be collected, a Perkin-Elmer Preparative Column (one inch diameter - Column "F") was employed. The same techniques of collecting samples of each fraction were used and again negative results were obtained. A sample gas cell which was adapted to the spectrophotometer and to the gas sampling device on the vapor fractometer was constructed so that a transfer of the gas was not necessary. Collection of each volatile fraction into this cell with subsequent subjection to the spectrophotometer produced no response.

Analysis of the gas collected from the fractometer, as a result of injecting one ml. of ethyl alcohol, indicated that identification was possible when unknown components were present in sufficient quantities. Since analysis of each volatile fraction contained in several two ml. samples of the benzene extract gave no response from the spectrophotometer, it was concluded that these volatile fractions were present in such small concentrations that it was not feasible to detect their presence by this method. Luers (28) found a similar problem in the study of fermenting beverages. He partially solved the problem by employing very large quantities of starting materials.

Summary of Techniques. Of the techniques investigated, paper electrophoresis appeared to have the least merit because separation of the carbonyl derivatives was not evident. Paper chromatography was found to be useful in the study of compounds released during fermentation and could probably be

used efficiently to study the compounds remaining in the pre-ferment if large volumes of starting materials were employed. Although paper chromatography might conceivably be used in this manner, gas chromatography appears to have greater merit for such studies. The results of these investigations indicate that gas chromatography was the only technique having sufficient sensitivity to detect compounds produced in trace amounts during fermentation. Further observations indicated that the use of varied columns, instrument conditions, and methods of sampling allowed the isolation of compounds present in concentrations less than .05 percent.

Collection of samples suitable for injection into the fractometer was probably the most critical problem encountered. Direct extraction appeared to be the best method of collection, but improvement of this technique should be emphasized in future experiments.

Identification of compounds separated by the vapor fractometer was the most difficult problem to solve. The method of adding a supposed constituent to confirm the identity of specific peaks was successful. This technique is limited, however, because it is possible for two compounds to have the same retention time.

Results indicated that the infra-red spectrophotometer was probably the most authoritative means of identifying trace volatile organic fractions of pre-ferments. However, these trace fractions must be concentrated before this method of identification is attempted. Further observations indicated that 500 ml. of the benzene extract would be necessary to produce sufficient quantities of the volatile fractions to allow identification by this method.

Future research should be directed toward the use of larger amounts of pre-ferments and study of less volatile materials until specific techniques and procedures are developed.

SUMMARY AND CONCLUSIONS

The volatile organic compounds produced during fermentation have been studied after collection of samples by three different methods. In addition, several means of separation and identification of the volatile organic fractions were investigated. From these studies the following information has been obtained:

It was shown that acetaldehyde was the only carbonyl compound released during fermentation in quantities large enough to be detected by paper chromatography.

Gas chromatography was a more valuable tool than paper chromatography to study the volatile organic compounds present in a pre-ferment. Paper electrophoresis was unsatisfactory.

The most effective means of collecting a sample of the volatile organic fraction was by a direct extraction of the pre-ferment with a water-insoluble solvent. Benzene was shown to be the most effective solvent to accomplish this extraction.

Acetaldehyde, ethyl alcohol, diacetyl, lactic acid and ethyl acetate were shown to be present in the pre-ferment after fermentation.

The volatile components contained in the benzene extract were present in the following amounts: acetaldehyde, .019 ml/1000 ml. of pre-ferment; ethyl alcohol, .062 ml/1000 ml. of pre-ferment; ethyl acetate, .037 ml/1000 ml. of pre-ferment; diacetyl, .035 ml/1000 ml. of pre-ferment; and lactic acid, .060 ml/1000 ml. of pre-ferment.

Small concentrations of the volatile compounds precluded conclusive identification work with infra-red spectrophotometer.

Diacetyl was increased during fermentation when Aerobacter aerogenes or Pediococcus cerevisiae were added to the pre-ferment.

SUGGESTIONS FOR FUTURE STUDY

Volatile organic compounds produced during yeast fermentation were studied by three methods. From these investigations it is evident that combinations of various methods of separation and identification must be used in future endeavors in this field of research.

The best starting point will be with much larger amounts of pre-ferments than were employed in this work.

Gas chromatography should provide the most useful tool for separating the volatile fractions of the pre-ferments and future investigations should be designed to further simplify the collection of samples suitable for injection into various columns. The Perkin-Elmer Preparative Column (one inch diameter) will probably be the most efficient means of collecting sufficient quantities of samples so that verification of unknown compounds may be accomplished.

Some means of identifying the fractions separated by the vapor fractometer will be necessary. Either the Mass Spectrograph or the Infra-red Spectrophotometer should offer the best possible solution for this problem.

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AN INVESTIGATION OF THE VOLATILE ORGANIC
COMPOUNDS PRODUCED DURING FERMENTATION

by

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The volatile organic compounds produced during fermentation of a pre-ferment were studied by three different analytical techniques. Major emphasis was placed on carbonyl compounds.

Paper electrophoresis of the 2,4-dinitrophenylhydrazones of carbonyl compounds released during fermentation offered no positive results. Paper chromatography of these derivatives showed that acetaldehyde was released during fermentation and gas chromatography of pre-ferment extracts confirmed the escape of acetaldehyde and indicated that ethyl alcohol and ethyl acetate were released in trace amounts.

Investigations concerning the separation of compounds present in the pre-ferment after fermentation showed that benzene and ethyl ether extractions of a yeast-free pre-ferment were more efficient means of obtaining samples of the volatile organic fractions than a vacuum distillation of the pre-ferment.

Qualitative and quantitative analysis of the benzene and ether extracts by means of a Perkin-Elmer Model 154-C Vapor Fractometer revealed the presence of acetaldehyde (.03 percent), ethyl alcohol (.1 percent), ethyl acetate (.06 percent), diacetyl (.06 percent) and lactic acid (.1 percent). Identification work was accomplished by adding micro amounts of supposed constituents and observing an increase in peak intensity (23). The use of an infra-red spectrophotometer and various sampling techniques were not effective means of identifying compounds present in concentrations less than .1 percent.

Studies involving the effects of microorganisms on the volatile fractions indicated that pre-ferments inoculated with Aerobacter aerogenes or Pediococcus cerevisiae produced greater amounts of diacetyl.